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Application News

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High Performance Liquid Chromatograph Nexera™
Inductively Coupled Plasma Mass Spectrometer ICPMS-2050

Multifaceted Evaluation of IgG Glycan Profiles Considering the Components in Glycosyltransferase Reactions

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User Benefits

- By combining multiple analysis methods, it becomes possible to conduct a multifaceted analysis of components related to glycosyltransferase reactions – sugars (substrate precursor), cofactors (metal ions), substrates (sugar nucleotides), and products (glycans).
- It is useful for developing culture process conditions to achieve the desired glycan profile.

■ Introduction

Glycans, also referred to as the "third life chain" alongside nucleic acids and proteins, are gaining attention for their roles in cell functions and biopharmaceuticals. *N*-linked glycans bound to antibodies can impact the quality of pharmaceuticals, including their efficacy and safety. For example, An increase in core fucosylated glycans enhances Antibody-Dependent Cellular Cytotoxicity (ADCC) activity, and an increase in terminal galactosylated glycans contributes to the improvement of Complement-Dependent Cytotoxicity (CDC) activity. Therefore, glycan profiles are regulated as one of the Critical Quality Attributes (CQAs) , and the culture process is studied to stably produce antibodies with the desired glycans.

For an efficient development of culture conditions aiming at controlling the glycan profile, strategic considerations focusing on the mechanism of glycan formation are required. Hence, in this article, we focused on glycosyltransferase reactions (Fig. 1), and analyzed multiple components such as substrate precursors (sugars), cofactors (metal ions), and substrates (sugar nucleotides). We introduce a case where a multifaceted evaluation was conducted, from input to output, in glycan formation.

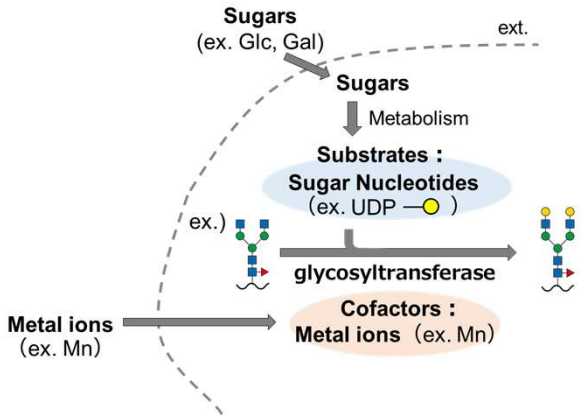

Fig. 1 Schematic diagram of the glycosyltransferase reaction
Glc：Glucose、Gal：Galactose

■ Cell culture focusing on galactosylation.

Cell culture condition

In this study, we especially focused on galactosylation. Galactose (substrate precursor) and Mn (cofactor) were supplemented to the culture medium under the conditions listed in Table 1. We conducted flask-fed batch culture using IgG producing CHO-K1 cells (Biological replicates n=3). As shown in Fig. 2, we regularly conducted sampling during the culture and collected the supernatant and cell pellets by centrifugation. The culture supernatant was used for the analysis of sugars, glycans, and metal ions, while the cell pellets were used for the analysis of sugars and sugar nucleotides.

Table 1 Cell culture condition

	Condition 1 (Control)	Condition 2 (+Mn)	Condition 3 (+Mn/+Gal)
Mn	Not supplemented (0.7 µM)	40 µM	
Gal	Not supplemented (0 g/L)		5 g/L (final conc.)
Seeding density	2.0×10^5 cells/mL		
Agitation	140 rpm		
Tmp. / Hum. / CO_2	37 ˚C / 80 % / 5 %		
Feed	D-Glucose Started when it dropped below 2 g/L and added every 24 hours so that the final conc. became 4 g/L.		

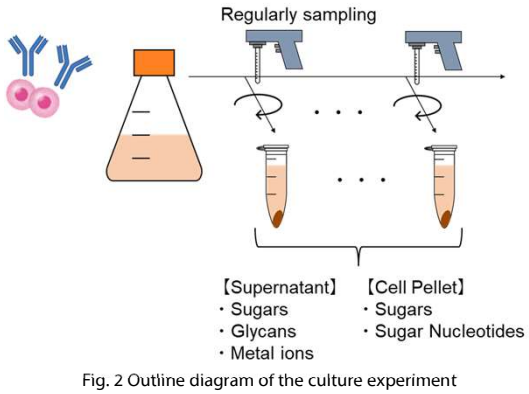

Fig. 2 Outline diagram of the culture experiment

Cell culture results

Viability and Viable Cell Concentration (VCC) were measured using an automated cell analyzer Vi-CELL BLU with trypan blue staining (Beckman Coulter, Inc.). Fig. 3 shows the change in Viability and VCC over time. The Viability and VCC were the same for both Mn/Galactose supplementation and control. This result indicates that the cell culture with Mn/Galactose supplementation could be performed without cytotoxicity.

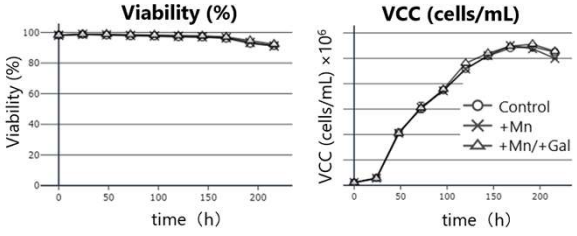

Fig. 3 Change in Viability and VCC over time.

■ Pretreatment and analytical conditions for culture samples

Metal ion analysis in culture supernatant (ICP-MS)

As shown in Fig. 4, the culture supernatant was diluted 20-fold with a mixed aqueous solution containing 1% (v/v) nitric acid and 0.5% (v/v) hydrochloric acid to prepare the analytical samples.

ICPMS-2050 was used to measure the Mn concentration in each sample under the same analytical conditions as Application News 01-00712 (Table 2)[2].

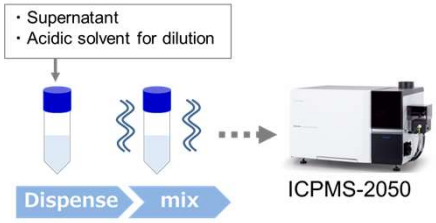

Fig. 4 Schematic diagram of the pretreatment for metal ion analysis in culture supernatant

Table 2 Analytical conditions for metal ion analysis

System	: ICPMS-2050
RF power	: 1.20 kW
Sampling depth	: 7.0 mm
Plasma gas flow rate	: 9.0 L/min
Auxiliary gas flowrate	: 1.10 L/min
Carrier Gas Flowrate	: 0.60 L/min
Dilution Gas Flowrate	: 0.25 L/min
Pump Speed	: 15 r.p.m.
Collision / Reaction Gas	: He / H_2

Sugar analysis in culture supernatant (LC-MS/MS)

To remove proteins from the culture supernatant, the supernatant was mixed with acetonitrile and centrifuged as shown in Fig. 5. The collected supernatant was diluted 10-fold and used as the analytical sample.

The relative concentration of galactose in each sample was measured using LCMS-8060 under the analytical conditions registered in LC/MS/MS Method Package for Sugars and Sugar Nucleotides.

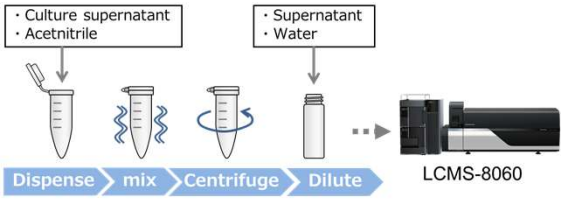

Fig. 5 Schematic diagram of the pretreatment for sugar analysis in culture supernatant

Intracellular sugar / sugar nucleotide analysis (LC-MS/MS)

Intracellular components were extracted from the cell pellets according to the instruction manual of the LC/MS/MS Method Package for Sugars and Sugar Nucleotides. Briefly, the cell pellets were suspended in cold solvent immediately after sampling to quench the cellular metabolism. The intracellular components were then extracted with methanol or water. After solvent drying using a centrifugal evaporator, the sample was re-dissolved in water and used as the analytical sample (Fig. 6).

The relative concentrations of galactose and UDP-galactose (the substrate for galactosyltransferase) in each sample were measured using LCMS-8060 under the analytical conditions registered in the LC/MS/MS Method Package for Sugars and Sugar Nucleotides*1.

*1. The analytical conditions for sugars and sugar nucleotides are different.

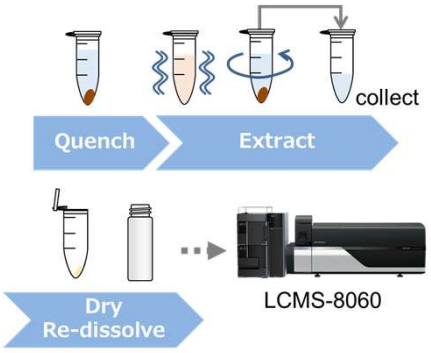

Fig. 6 Schematic diagram of the pretreatment for intracellular sugar / sugar nucleotide analysis

Glycan analysis of antibodies in culture supernatant (HPLC-RF)

Antibody purification in the culture supernatant, the release of the glycans from the antibodies, and the fluorescent labeling of the glycans were performed using the EZGlyco mAb-N Kit with 2-AB (Sumitomo Bakelite Co. Ltd.). A summary of the pretreatment process using this kit is shown in Fig. 7. The amount of antibody used in this study was ca. 3-10 µg.

Nexera XR was used for glycan analysis under the analytical conditions shown in Table 3. The result of the reference glycan standard samples is shown in Fig. 8.

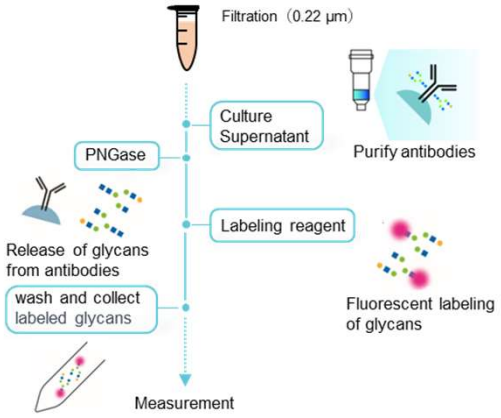

Fig. 7 Pretreatment using the EZGlyco mAb-N Kit with 2-AB

Table 3 Analytical conditions for glycan

System	: Nexera XR
Column	: Shim-pack™ GIST-HP Amide [Metal free] (100 mm × 2.1 mmI.D.,1.9 µm)*2
Mobile phases	: A) 100 mM ammonium formate (pH4.4)
	: B) Acetonitrile
Time program	: B Conc.78% (0 min)→69% (50 min)→ 50% (50.01-55 min)→78% (55.01-62 min)
Column temp.	: 40 ˚C
Flow rate	: 0.5 mL/min
Injection Volume	: 1 µL
Detection	: Fluorescence (Ex. 330 nm, Em. 420 nm) (using RF-20Axs, semi-microcell)

*2. P/N : 227-30951-02

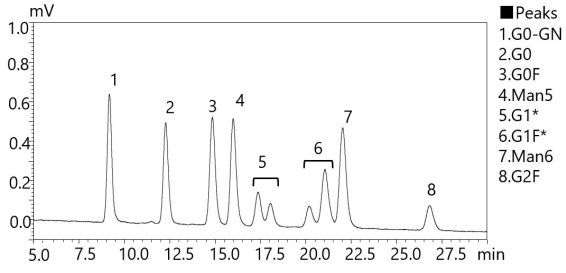

Fig. 8 Chromatogram of the glycan standard sample
Peaks 5 and 6 include the structural isomers of Galactose (G1：G1a, G1b、G1F：G1Fa, G1Fb)

■ Analytical results of various components involved in galactosyltransferase reaction

Results of Mn analysis in culture supernatant

As the initial concentration of Mn is more than 50 times different under the conditions with and without Mn addition, the time-course changes when the initial concentration was set to 0 were shown in Fig. 9. The Mn uptake rates per cell were calculated from the measurement values at 0 h and 264 h, and are shown in Fig. 10. Fig. 9 and 10 show that more Mn was taken up into the cells under the Mn-supplemented condition (Mn only or Mn/ Galactose co-supplemented) than under the control condition.

In the samples cultured for 96 hours under the condition where Mn/Galactose were co-supplemented (+Mn/+Gal), a high measurement value was recorded. This value was considered an error in the sample preparation process because all other elements measured simultaneously showed the same trend, except for the internal standard element.

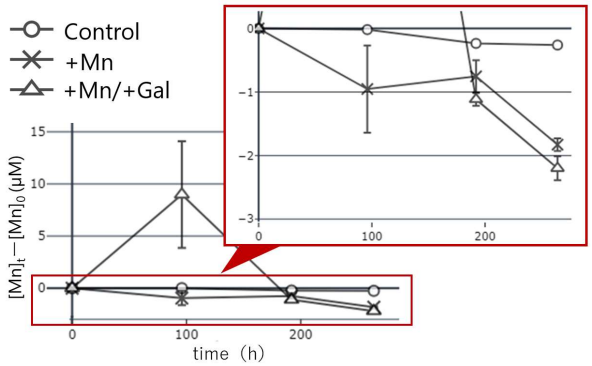

Fig. 9 Time-course changes in Mn concentration
(displaying the difference from the initial concentration)

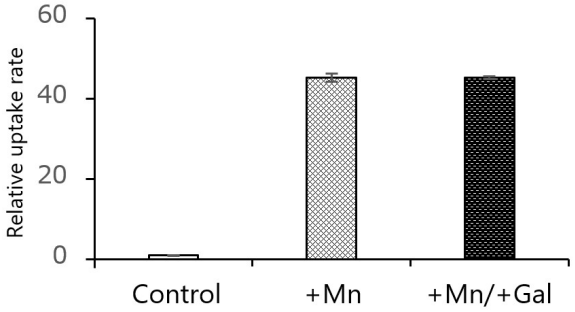

Fig. 10 Mn uptake rate per cell (represented as 1 for the Control)

Results of intracellular and extracellular sugars and sugar-nucleotides analysis

Examples of the mass chromatograms are shown in Fig. 11 and 12. Galactose and UDP-galactose were able to separate from each isomer by using the LC/MS/MS Method Package for Sugars and Sugar Nucleotides.

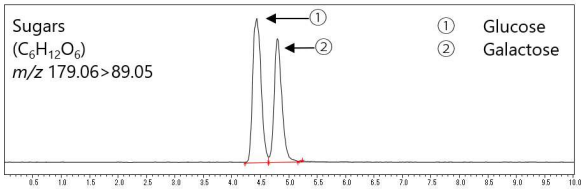

Fig. 11 Mass chromatogram of a culture supernatant sample

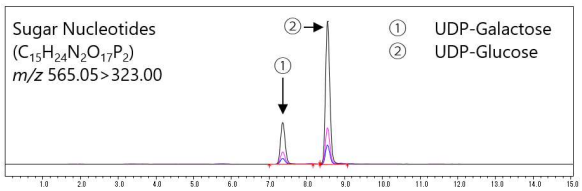

Fig. 12 Mass chromatogram of an intracellular sample

The time-course changes in galactose and UDP-galactose are shown in Fig. 13. It was demonstrated that galactose was consumed and converted to UDP-galactose (the substrate for galactosyltransferase reaction).

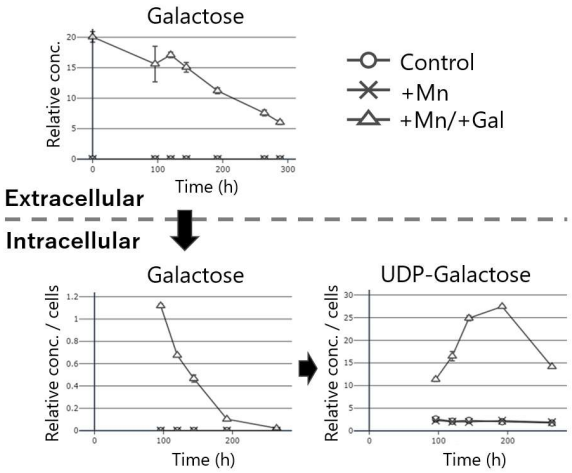

Fig. 13 Time-course changes in sugars and sugar nucleotides

Glycan profiles of antibodies in culture supernatant

Glycan profiles between three conditions were compared for samples in the growth and stationary phases (Fig. 14). The results showed that the ratio of galactosylated glycans, G1F*3 and G2F, increased in the order of +Mn/+Gal > +Mn > Control. Furthermore, the ratio of galactosylated glycans was maintained even in the stationary phase only under +Mn/+Gal condition.

*3. G1F : The sum of G1Fa and G1Fb

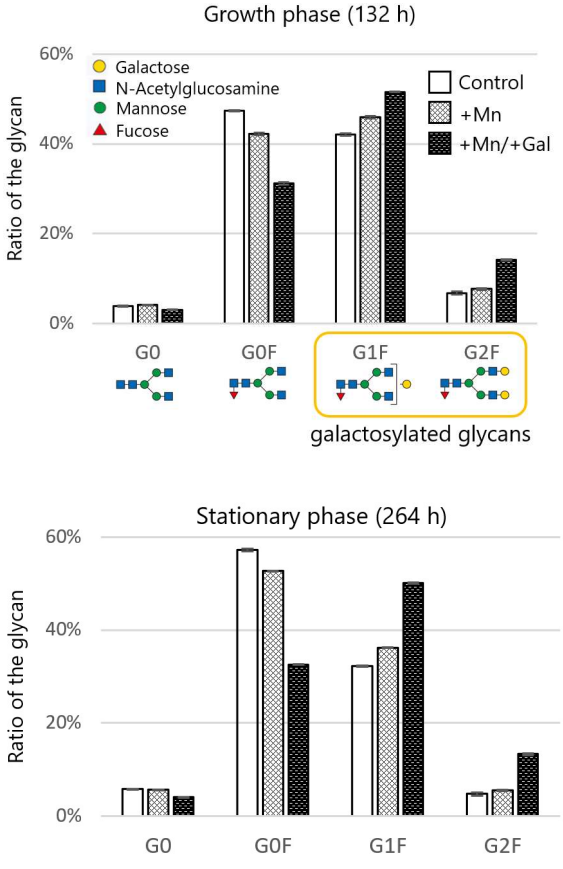

Fig. 14 Glycan profiles of antibodies in culture supernatant

■ Conclusion

In this article, we focused on the glycosyltransferase reaction involved in the formation of *N*-linked glycans of antibodies, and analyzed samples cultured with the supplementation of substrate precursors (sugars), cofactors (metal ions), and substrates (sugar nucleotides). Particularly this time, we focused on the galactosylation, and analyzed multi-components such as galactose, Mn, and UDP-galactose as substrate precursor, cofactor, and substrate, respectively.

We obtained data showing that the substrate precursor (galactose) and enzyme cofactors (Mn) are taken into the cell from medium, and they promote the formation of galactosylated glycans.

Although controlling the glycan profile of antibodies is a challenging task, by analyzing the multiple components involved in the glycosyltransferase reaction, we can understand the reasons for changes in the glycan profile, enabling the strategic development of culture conditions for controlling the glycan profile.

<Acknowledgements>

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<References and Related Applications >

1) Wang, Ziyan, Jianwei Zhu, and Huili Lu. 2020. "Antibody Glycosylation: Impact on Antibody Drug Characteristics and Quality Control." *Applied Microbiology and Biotechnology* 104 (5): 1905–14.
2) Analysis of Metal Elements in Culture Medium Using ICPMS-2050 (01-00712)

■ Related Products

- **LC/MS/MS Method Package for Cell Culture Profiling**
- **LC/MS/MS Method Package for Primary Metabolites**

Sugars are also used in energy metabolism and the pentose phosphate pathway. Further understanding of sugar metabolism is expected to be provided by the analysis of metabolic components. As shown in Fig. 15, LC/MS/MS Method Package for Cell Culture Profiling and Primary Metabolites enhance the intra/extracellular multi-component analysis related to sugar metabolism.

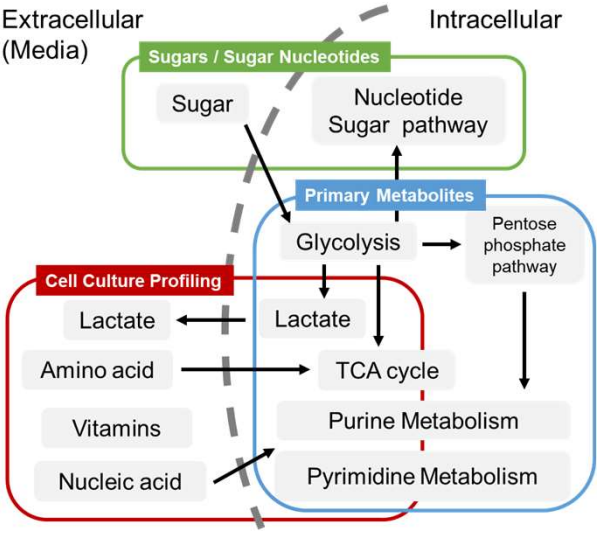

Fig. 15 LC/MS/MS method packages related to sugar metabolism.

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